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Research Article

Participation of NO-synthase in Control of Nitric Oxide Level in Rat Hippocampus after Modelling of Ischaemic and Haemorrhagic Insult

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Abstract

Electron paramagnetic resonance (EPR) was used as a method for recording the content of the nitric oxide (NO) in hippocampal tissues of intact rats and rats after modelling of ischaemic and haemorrhagic stroke. Based on direct measurements of NO by EPR spectroscopy, it was shown that, within 5 hours after the onset of symptoms of ischaemic and haemorrhagic stroke, the formation of NO in the hippocampus was reduced by a factor of 2-3 and this reduction was maintained for a period of between 24 and 72 hours. The results show that a systemic character of a decrease in the intensity of NO production during the modelling of ischaemic events in the brain reflects the effects of central dysregulation of the functions at the level of the whole organism such that it is appropriate to consider implementing the correction of the vital systems of the body in a stroke. It has indicated that non-selective NO-synthase blocker L-NAME reduced the low level of NO production by a factor of 3 by its administration within 72 hours after post-ischaemic and

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haemorrhagic stroke. It was discovered however that L-NAME returns the level of NO production to baseline (control) by its administration within 5 hours after ischaemia.

Keywords

Nitric oxide, Electron paramagnetic resonance, Spin trap, Ischaemic brain stroke, Haemorrhagic brain stroke

Introduction

There appears to be increasing evidence about the impact of regulatory substances of intestinal microflora on the functional state of the brain of animals (Luczynski et al. 2016). Under normal conditions, the symbiosis of the organism and the microflora is essential for the course of natural processes in the body and its adaptation to a changing environment. However, in the development of pathological processes, commensal bacteria (normal microflora) begin to play an important role in the pathogenesis of systemic and local inflammation, in the changes in blood flow and in the disorder of plasticity in the nervous tissue which are all reflected in the shift in the balance of neurotransmitters towards predominance of the excitation or inhibition and eventually accompanied by breakdown of the control of functions (Matta Guedes et al. 2016). Uncontrolled formation of anti-inflammatory substances affects the nitric oxide (NO) system which is one of the key regulators of the intra- and extracellular processes in normal and pathological conditions. It is known that the functioning of the tissues depends on a number of key factors. One of these is the necessity for a sufficient amount of oxygen to be delivered through the bloodstream to maintain the oxidative processes. The prolonged deficiency of oxygen leads to hypoxia of the brain (Bolaños and Almeida 1999, Müller Hoff et al. 2017) which is, under certain conditions, accompanied by the development of tissue ischaemia. This occurrc in a case of disruption in the supply of oxygen to tissues required due to the natural process of biological oxidation; it is an important component of the pathogenesis of many diseases (Manukhina et al. 1999Donnan et al. 2008). Such disruption may occur in post-traumatic stress disorder (Campos et al. 2013), characterised by memories of the original traumatic event. Interruption of cerebral blood flow decreases the oxygen supply to the brain and also leads to brain ischaemia which can result in an ischaemic stroke, followed by damage to the brain tissue and its functions (Donnan et al. 2008, Doyle et al. 2008, Lambertsen et al. 2012). Interruption of the oxygen supply to the brain also occurs as a result of vessel thrombosis or ruptured aneurysm which often leads to ischaemic or haemorrhagic stroke (Liu et al. 2015). In these pathological processes, NO plays both protective and destructive roles which are determined by many factors which define the start of the various processes of NO-synthases (Baider et al. 2009, Remizova et al. 2011, Wang et al. 2016). According to this study of the pathogenesis, methods of correction and the mechanisms of stroke are important both from the theoretical and practical points of view. Nitric oxide is an important signalling molecule that is widely used in the nervous system. Taking account of its roles in synaptic plasticity and elucidation of calcium-dependent, NMDAR-mediated activation of

nitric oxide synthase (NOS), numerous molecular and pharmacological tools have been used to explore the physiology and pathological consequences for nitrergic signalling (Steinert et al. 2010). The participation of NO in the mechanisms for development of various pathological conditions has been attracting great interest (Steinert et al. 2010, Pacher et al. 2007, Terpolilli et al. 2012). NO typically exerts its physiological functions by binding to the iron (Fe^{2+}) of heme or by S-nitros(yl)ation of proteins but it also participates in a number of other biochemical reactions (Vanin et al. 2002, Hansen and Jensen 2010, Hill et al. 2010). By activating heme-containing soluble guanylate cyclase and the ADP-ribosyltransferase, the NO is involved in regulating the intracellular concentration of Ca^{2+} ions and in the regulating of the pH at the cerebral ischaemia (Evgenov et al. 2006, Erusalimsky and Moncada 2007, Borodulin et al. 2013).

In the vital functions of animals, the role of NO is especially significant in the cardiovascular function (Reutov et al. 2007, Andrianov et al. 2008) and in nervous systems (Steinert et al. 2010, Terpolilli et al. 2012, Gainutdinov et al. 2011). At the present time, the development of cerebral ischaemia and the following stroke is associated with impaired cerebral blood flow, as well as violations of its regulation by the NO system (Remizova et al. 2011, Terpolilli et al. 2012). NO, synthesised by constitutive isoforms of NO-synthases (NOS), provides an adequate blood supply to the brain regions, affects the activity of neurons and regulates cell metabolism (Forstermann and Sessa 2011). On the other hand, there is no doubt that the role of the NO system in the pathogenesis of a number of diseases associated with vascular disorders is defining (Pacher et al. 2007, Terpolilli et al. 2012, Godínez-Rubí et al. 2013). Previously, by the methods of EPR spectroscopy, our team has evaluated the effect of ischaemic stroke on the intensity of NO production in the tissues of the brain, heart and liver of rats *in vivo* (Andrianov et al. 2016). It was shown that within 5 hours after modelling of ischaemia, a double reduction of NO production was observed in the tissues of the hippocampus, heart and liver and it was maintained for 3 days. Subsequently, we studied the intensity of NO production in rat hippocampal tissue by EPR spectroscopy using spin-trapping techniques at the haemorrhagic stroke simulation by administering autologous blood to the brain. This investigation continues the previous research and its main purpose is to study the processes of NO-synthase involvement in the control of NO levels in the hippocampus of rats after modelling both ischaemic and haemorrhagic stroke.

Material and Methods

Experimental protocol. Simulation of ischaemic and haemorrhagic stroke in rats and application of trapping for nitric oxide

Modelling of ischaemic and haemorrhagic stroke was produced on rats in the Institute of Physiology of NAS of Belarus, Minsk. Animals were kept in standard vivarium conditions (12/12- light and dark rhythm, air temperature of 23±1°C and stable supply and exhaust ventilation) with free access to water and food (*ad libitum*) and a diet in accordance with the standards for keeping laboratory animals. For the modelling of ischaemic stroke, animals

were subjected to 5-minute hypoxia (conditional rise to a height of 4500m above sea level which corresponded to a pressure of 432mmHg and a decrease in oxygen partial pressure pO_2 from 159mmHg to 90mmHg on average) (Coupé et al. 2013, Kulchitsky et al. 2014). After 5, 24 and 72 hours, it was carried out fence of the hippocampus. A similar extraction of tissue samples was also produced from control animals. All experiments on animals were conducted in accordance with the rules of the Ethics Committee of the Institute of Physiology of the National Academy of Sciences of Belarus. Protocols of experiments correspond to international normative ethical documents. In experiments of modelling haemorrhagic stroke, the head of anaesthetised animals was fixed in a stereotaxic apparatus in such a way that lambda and bregma were located in the same horizontal plane (Roch et al. 2007). After treatment of the skin with 5% solution of iodine and subcutaneous injection of 2% solution of lidocaine, the soft tissue of the calvarias was dissected, the periosteum was removed (after additional injections under the periosteum of 2% solution of lidocaine) and haemostasis was carefully conducted. A hole with a diameter of 2.5-2.8mm was drilled using a microdrill in the skull bone under stereotactic coordinates with the right caudal to bregma 5.0mm lateral to the midline and 5.0mm. The dura was carefully opened and haemostasis was performed. Through a burr hole into the brain using a micromanipulator, a glass pipette was immersed to a depth of 4.5-5.0mm (tip diameter 2.0mm) and 40nl of the autologous blood was injected (data coordinates and depth of insertion micromanipulator correspond with hippocampal CA1 region). The removal of the hippocampus was performed in rats treated with autologous blood and in rats which did not have the injection into the brain after 5, 24 and 72 hours. For the whole series of experiments, on the day of the experiment, rats were anaesthetised by intraperitoneal injection of a mixture of ketamine-chloralose-acepromazine (55.6mg, 5.5mg and 1.1mg/kg, respectively). The components of the spin trap NO (DETC-Na, $FeSO_4$, sodium citrate) were injected 30 minutes before the extraction of studied tissue. The fragments of the hippocampus with size 1.5x1.5mm were isolated immediately after decapitation of the rats and immediately frozen at liquid nitrogen temperature. The studies of the effect of non-selective blocker of NO-synthase (NOS) on the NO production in the modelling of ischaemic and haemorrhagic strokes were carried out to estimate the contribution of different sources of NO. The L-NAME in a dose of 10mg/kg was administered intraperitoneally 60 minutes prior to decapitation.

Formation of the complex of NO with the spin trap in rat tissues

The difficulty in determining the maintenance of the free NO in the tissues of the organism is due to its short lifetime which appears in low concentrations in tissues. Recently, one of the most effective methods for the detection and quantification of NO in biological tissues is the method of electron paramagnetic resonance (Vanin et al. 2002Khramtsov and Volodarsky 1998). Using the technique of spin traps, it allows the detection of NO in low concentrations (Mikoyan et al. 1997). We used the complex of Fe^{2+} with diethyldithiocarbamate ($(DETC)_2$-Fe^{2+}) as a spin trap. The complex of the spin trap with NO ($(DETC)_2$-Fe^{2+} -NO) is characterised by easily recognisable EPR spectrum at the value of the g-factor g = 2.038 and triplet hyperfine structure. DETC-Na was intraperitoneally administered at the dose of 500mg/kg in 2.5ml of water. Solution mixture: ferrous sulphate

($FeSO_4$ × 7 H_2O, Sigma, USA) at a dose of 37.5mg/kg and sodium citrate at a dose of 187.5mg/kg (in 1ml water per 300g body weight) prepared immediately before administration and administered subcutaneously at three points: the right and left hip and in the rostral part of the interscapular region. Details of the experiment and method have been described previously (Andrianov et al. 2016Gainutdinov et al. 2013). The weight of the samples was 100mg. Tissue samples were immediately frozen in liquid nitrogen and transported under frozen conditions from Minsk to Kazan. In this state, the complex spin trap with NO ($(DETC)_2$-Fe^{2+} -NO) was well preserved and the signal from the complex did not change for a month (Vanin et al. 2002, Andrianov et al. 2016, Mikoyan et al. 1997). The measurements of the spectra of the complex $(DETC)_2$-Fe^{2+}-NO were performed on the spectrometer ER 200 SRC Bruker in the X band (9.50 GHz) with the modulation of the magnetic field of 100kHz, the modulation of the amplitude of 2Hz, the microwave radiation power of 30mW, time constant of 200ms and the temperature of 77ºK in the Finger Dewar Bruker. The amplitude modulation, the amplification and the RF power in all experiments were chosen under the conditions of absence of over-modulation and saturation of the EPR signal and remained the same for all measurements. The amplitude of the EPR spectra were always normalised to the weight of the sample and the amplitude of the EPR of the signal of the reference sample (details of the EPR signal measurement technique described earlier (Gainutdinov et al. 2011).

Statistical processing of experimental results

The results are shown as mean ± SEM. The unpaired Student's t-test and non-parametric Mann–Whitney test were used for comparison between two groups. One-Way ANOVA followed by the Tukey post-hoc test and a repeated Two-Way ANOVA were used for comparison between statistical groups. The statistical software SigmaStat32 was used. The statistical significance criterion was $p<0.05$.

Results

EPR spectra of the hippocampus of intact rats and rats after modelling of haemorrhagic stroke

Fig. 1 shows the EPR spectrum of the hippocampal tissue of intact rats. In this spectrum, there is a typical triplet signal from the complex $(DETC)_2$-Fe^{2+}-NO at the value of the g-factor, equal to 2.038 (Mikoyan et al. 1997). Moreover, the signal of the $(DETC)_2$-Cu complex is presented in the same area. At the bottom of the figure, the EPR spectra of the hippocampal tissue within 5 hours after modelling of the haemorrhagic stroke is shown. The solid line represents the sample range, the dashed line shows the signal from the nitric oxide associated with the spin trap, as part of the spectrum of the $(DETC)_2$-Fe^{2+}-NO complex. The relative change of the amount of the NO-containing complexes were evaluated by the integrated signal intensity of the spin trap, $(DETC)_2$-Fe^{2+}-NO.

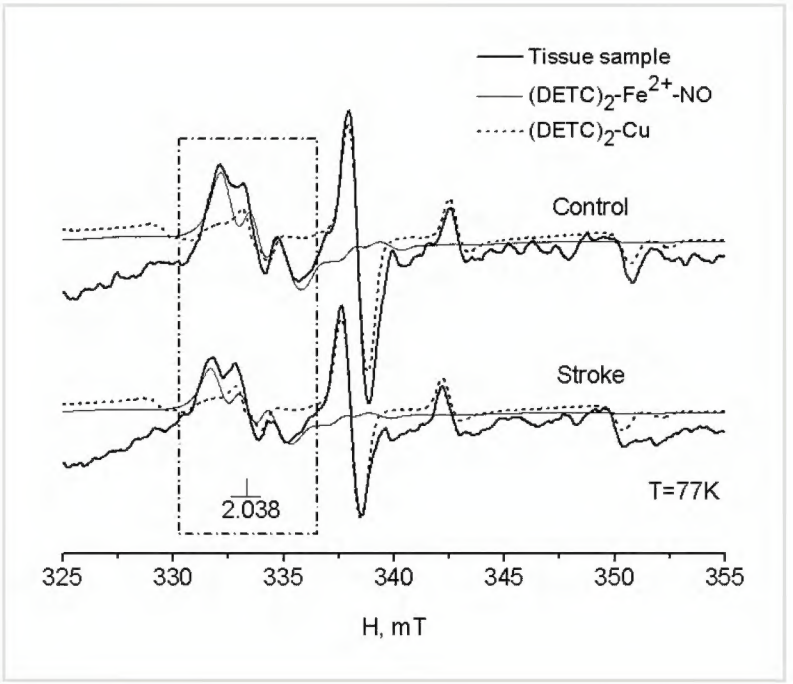

Figure 1.

EPR spectra of hippocampus of healthy rat and rat after haemorrhagic stroke. The signals from: a) tissue sample, b) complex $(DETC)_2$-Fe^{2+}-NO. Temperature is 77°K. The rats were injected with $(DETC)_2$-Fe^{2+} - citrate. g = 2.038.

Dynamics of the maintenance of NO in the hippocampus after modelling ischaemic and haemorrhagic stroke

Fig. 2 shows the statistical data of the integrated signal intensity of $(DETC)_2$-Fe^{2+}-NO in the hippocampus spectra representing the effects of the haemorrhagic stroke on the NO production in the hippocampus tissues at different stages after the stroke. There is a decrease by a factor of 3 of the NO production in the hippocampus within 5 hours after modelling of the haemorrhagic stroke. After 72 hours (three days), the level of NO production in the hippocampus remained reduced by a factor of 2.

Fig. 3 shows the statistical data of the integrated signal intensity of $(DETC)_2$-Fe^{2+}-NO in the hippocampus spectra representing the effects of the ischaemic stroke on the NO production in the hippocampus tissues at different stages after stroke. The results of the analysis show a significant decrease of the NO production after modelling of the ischaemic stroke. It can be seen that the NO maintenance in the hippocampus has decreased by 2-3 times after 5 hours. Moreover, this decrease of the NO production has remained for 24 and 72 hours after the stroke.

The dynamics of the NO maintenance in the hippocampus after modelling of the ischaemic and haemorrhagic stroke with administration of the NO synthase blocker L-NAME

Taking into account that the NO may play a pathogenic role in a number of pathological conditions of the nervous system, including ischaemia, some NOS inhibitors have become the subject of intensive study as potential neuroprotective agents (Forstermann and Sessa

2011, Zaripova et al. 2014). Hence, the next step in our research was to study the effect of the NOS blockers on the NO production in the model of the haemorrhagic stroke. L-NAME was used at a dose of 10mg/kg. The L-NAME was injected intraperitoneally 60 minutes before the decapitation as a non-selective NOS blocker. The results indicate that the nonselective blocker of NOS L-NAME reduced by 3 times the low level of the NO production by its administration within 72 hours after the ischaemic and haemorrhagic stroke (Figs 2, 3). However, it was found that the L-NAME returns the level of the NO production to the initial level after 5 hours of ischaemia.

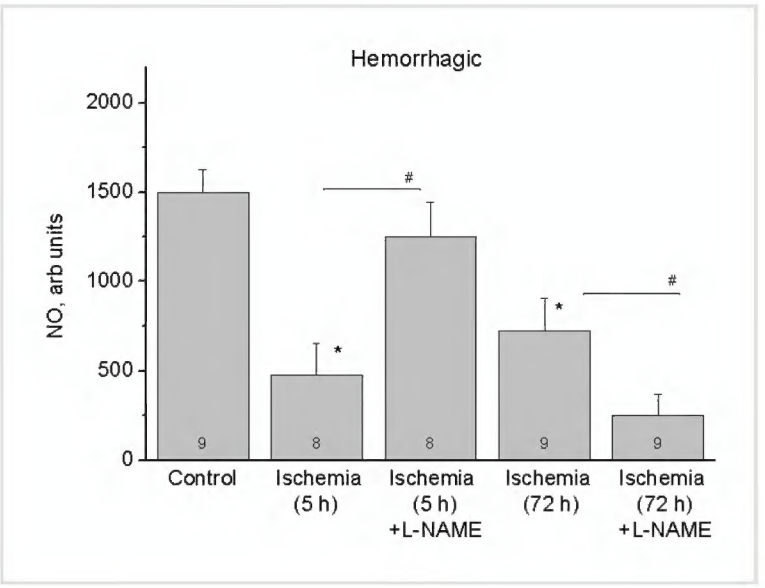

Figure 2.

The relative content of NO in the hippocampus of healthy rats (Control) and rats after 5 (Ischaemia, 5h) and 72 (Ischaemia, 72h) hours of the haemorrhagic stroke and also after using of inhibitor L-NAME (Ischaemia, 5h + L-NAME) and (Ischaemia, 72h + L-NAME) respectively. The ordinates axis is the average integral intensity of the signal.

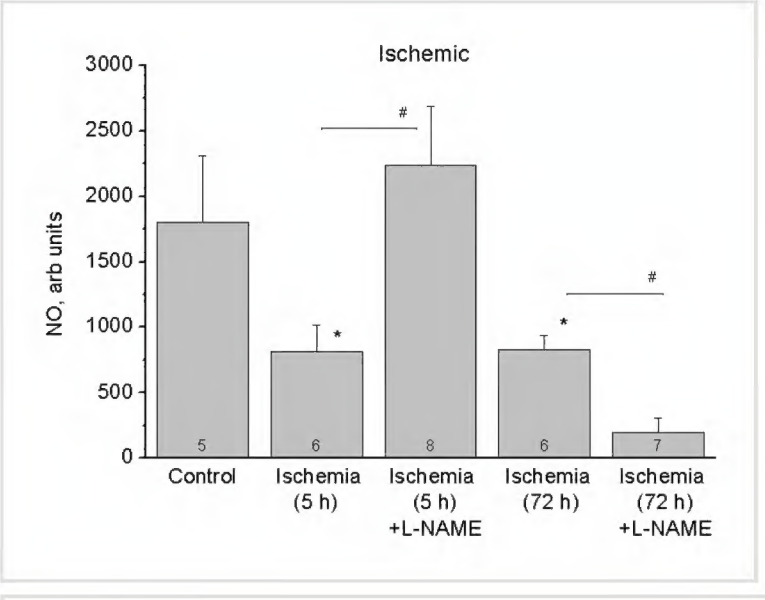

Figure 3.

The relative content of NO in the hippocampus of healthy rats (Control) and rats after 5 (Ischaemia, 5h) and 72 (Ischaemia, 72h) hours of the ischaemic stroke and also after using of inhibitor L-NAME (Ischaemia, 5h + L-NAME) and (Ischaemia, 72h + L-NAME) respectively. The ordinates axis is the average integral intensity of the signal.

Discussion

Discussion of the design of experiment

The problem of cerebral ischaemia is most acute in the world (Donnan et al. 2008, Lambertsen et al. 2012, Godínez-Rubí et al. 2013). Accumulating experience of observations shows that the ischaemic injury and inflammation account for the pathogenic progression of the stroke (Yilmaz and Granger 2008, Yang et al. 2011). The cascade of inflammatory reactions and molecular cellular processes that result in organ damage after ischaemic injury has been studied extensively. However, the extent to which the initial cellular injury contributes to propagation of the inflammatory response and further tissue damage is poorly understood. In the experiments, we modelled the ischaemic stroke and haemorrhagic stroke (Coupé et al. 2013, Kulchitsky et al. 2014, Roch et al. 2007). As the used method of applying a spin trap places some limitations on the experimental protocol, such as the need to wait for 30 minutes for the accumulation signal, we have not included in the research the short interval (up to 3 hours) from the beginning of the ischaemia.

Discussion of the results of experiment

At the present time, the development of cerebral ischaemia and the subsequent occurrence of stroke are associated with impaired cerebral blood flow, as well as with impaired regulation of the blood supply to the brain tissues by the system of NO (Bolaños and Almeida 1999, Remizova et al. 2011, Terpolilli et al. 2012). The role of NO in ischaemia development has long attracted the attention of researchers. Thus, using the method of the measurement of the NOS activity, it has been shown that the activity of the neuronal NOS increased within 10min after the start of the ischaemia of the brain and has maximum after 3 hours (Samdani et al. 1997) and that the expression of iNOS has started between 24 and 48 hours after ischaemia (Iadecola et al. 1997). With the use of EPR spectroscopy, it has been found that the NO production increased after 5 minutes of ischaemia and continued for 60min (Tominaga et al. 1994, Sato et al. 1994). The NO overproduction in acute hypoxia has also been noted (Manukhina et al. 1999). In a number of studies with some models of cerebral ischaemia, results were obtained which do not coincide with the point of view of the neurotoxic role of ischaemia generated NO. It has been shown that the use of inhibitors of NOS L-NNA and L-NAME does not reduce the size (volume) of infarction in a model of focal cerebral ischaemia in the rat (Dawson et al. 1992, Sancesario et al. 1994) and, on the contrary, increases the focal ischaemic stroke (Yamamoto et al. 1992). NO donors are used as neuroprotective agents at ischaemic injury (Evgenov et al. 2006, Godínez-Rubí et al. 2013). Based on the time course of ischaemia-induced changes in NO levels and NOS regulation in the brain and cerebral blood vessels, several strategies have been suggested to manipulate the NO system for the treatment of strokes (Bolaños and Almeida 1999). There are also results showing reduction of NO maintenance in the ischaemic cortex of the left hemisphere of rats after the modelling of ischaemic stroke (Gainutdinov et al. (2011)). Therefore, based on the time course of changes in the levels of NO and the regulation of NOS in the brain and the brain vessels induced by ischaemia, several strategies should be used to manipulate the NO system in the treatment of stroke (Terpolilli et al. 2012). Thus,

the dynamics of NO maintenance in the brain tissues during the occurrence and course of brain ischaemia remains unclear, despite the recognition of the fact that the main contribution is NO which is produced by nNOS and iNOS (Bolaños and Almeida 1999, Steinert et al. 2010). The NO is synthesised in response to physiological need with the help of the enzyme NO-synthase from its metabolic precursor, the amino acid L-arginine. Several isoforms of NOS are known; the nitro and alkyl L-arginine derivatives, i.e. structural analogues of the natural substrate of the enzyme (L-NAME, L-NNA et al.) is best known amongst a large number of the NOS inhibitors (Forstermann and Sessa 2011, Zaripova et al. 2014). Taking into account that the NO may play a pathogenic role in a number of pathological conditions of the nervous system, including ischaemia, some NOS inhibitors have become the object of intensive study as potential neuroprotective agents. Hence, the next step in our research was to study the effect of the NOS blockers on the NO production in the model of the haemorrhagic stroke. The results indicate that L-NAME reduced by a factor of 3 the low level of the NO production by its administration within 72 hours after the ischaemic and haemorrhagic stroke. However, it was found that the L-NAME returns the level of the NO production to the initial level after 5 hours of ischaemia. This result is extremely important and a possible explanation for this fact was obtained in the electrophysiological experiments. Such development indicates a violation of the conditions of interneuron communications and disrupting the input-output processes at the level of individual neurons and their populations at lower oxygen tension in the tissues of the hippocampus, which controls processes of storage and organisation of the behaviour (Coupé et al. 2013, Kulchitsky et al. 2014, Roch et al. 2007). The short-term effect of increasing the amplitude of excitatory postsynaptic potentials and population spikes on the output signal level can be explained by the development of excitotoxicity, with the release of excitatory amino acids. The obtained experimental data of the balance of input-output relationship in a population of hippocampal cells, after inhibition of NOS, indicate the advisability of inhibiting the excessive production of NO in situations involving the weakening of the delivery of oxygen to different parts of the brain.

Conclusion

Thus, the analysis of literature and the results of our experiments show the ambiguity of the data that reflects the well-known fact of dose-dependent effects of NO in the brain. In the present work, it was demonstrated that the process of the comprehensive approach and the application of precision methods for measuring NO levels made it possible to receive data for the dynamics of NO production in nerve tissue.

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Author contributions

Khalil L. Gainutdinov carried out research using electron paramagnetic resonance and formalised the results of this section of the study. He formulated the conclusions of the work. Svetlana G. Pashkevich conducted experimental studies in rats and formalised the results of the study. Vyatcheslav V. Andrianov carried out research using electron paramagnetic resonance and formalised the results of this section of the study. Guzel G. Yafarova carried out research using electron paramagnetic resonance and formalised the results of this section of the study. Margarita O. Dosina conducted experimental studies in rats and formalised the results of the study. Tatiana Kh. Bogodvid carried out research using electron paramagnetic resonance and formalised the results of this section of the study. Julia P. Stukach conducted experimental studies in rats and formalised the results of the study. Dinara I. Silant'eva carried out research using electron paramagnetic resonance and formalised the results of this section of the study. Aleksandra S. Zamaro conducted experimental studies in rats and formalised the results of the study. Timur V. Sushko conducted experimental studies in rats and formalised the results of the study. Vladimir Kulchitsky conducted experimental studies in rats and formalised the results of the study. He formulated the conclusions of the work.

Conflicts of interest

No potential conflict of interest was disclosed by any of the authors.

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